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14. ABSTRACT
Metastasis and tumor progression at metastatic sites ultimately results in the demise of prostate cancer (PCa) patients. Currently there are no highly effective methods that can target these problems. Aptamers, which have proven clinical efficacy for non-neoplastic disease and are generally more specific and stable than antibodies, may have clinical utility in PCa. However, defining aptamers that can prevent metastasis is challenging due to the fact that many proteins that play a role in the metastatic process are unknown. The overall goal of this project is to develop novel method to inhibit cancer metastasis. The major hypothesis to be tested is that aptamers (short oligonucleotides) can be developed that target the process of invasion, without prior knowledge of a target protein, and that these aptamers will inhibit the development of metastasis. We also hypothesize that the aptamers can be used to identify cell surface proteins that are important mediators of metastasis. This latter information is important as it may help identify further therapeutic targets. We have made some initial progress towards i

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INTRODUCTION:

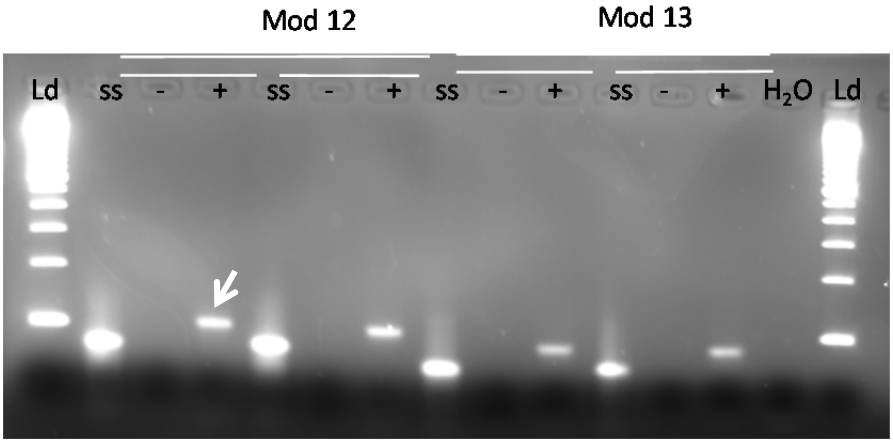
Progressive prostate cancer (PCa) is accompanied by metastasis. A major component of the metastatic cascade is the ability of cells to invade tissues. The overall goal of this project was to develop novel method to inhibit cancer metastasis. The major hypothesis to be tested was that aptamers (short oligonucleotides) can be developed that target the process of invasion, without prior knowledge of a target protein, and that these aptamers will inhibit the development of metastasis. We have made some initial progress towards testing this hypothesis. Specifically, we have identified RNA aptamers, using a novel application of a process called "systemic evolution of ligands by exponential enrichment" (SELEX), that bind prostate cancer (PCa) cells that we selected for their high metastatic ability. We had also determined that the aptamers functionally inhibit in vitro invasion and can inhibit development of PCa metastasis in a murine model. To bring these studies closer to potential clinical utility, we proposed the following aims: (1) Test the most frequently identified aptamers in their ability to inhibit cancer cell survival and invasion in vitro; (2) Determine the ability of aptamers with the greatest in vitro invasive inhibitory properties to inhibit the development of metastasis of PC-3 and C4-2B cell lines in both models in which tumor is implanted in the prostate or injected into the left ventricle; and (3) Identify the aptamers' cell surface target proteins using the aptamers in an affinity column.

BODY:

Aim 1

In order to perform this project, we needed to have high quality aptamer that can be administered in vivo. Our original plan was to purchase this from a company, but then they could not guarantee a feasible production amount (i.e. they did not guarantee a high yield). Thus we took on the challenge of synthesizing our own aptamer to ensure production quality and sufficient yield. We initially attempted to PCR amplify aptamer sequences from plasmid clones; however, even at 35 cycles we often found contaminant bands and very feint aptamer-specific bands, indicating low yield with possible contaminants. To overcome the problem of amplifying from a plasmid, we ordered full DNA sequences of the aptamer sequence alone to use as PCR template to create aptamer RNA synthesis template. We initially had to test a variety of PCR conditions to optimize the PCR to minimize contaminating non-specific PCR products, while maximizing specific target yield. This allowed us to perform just 5 cycles of PCR to create sufficient DNA template for RNA aptamer synthesis. We then needed to optimize the amount of DNA template to use, as our initial attempts indicated we were not using sufficient amounts; however, once we precipitated and concentrated the DNA templates, we were able to perform RNA transcription that yielded good amounts of uncontaminated RNA aptamer (Fig. 1). Unfortunately, the process to get to this final success point, including the unsuccessful attempts took 6 months.

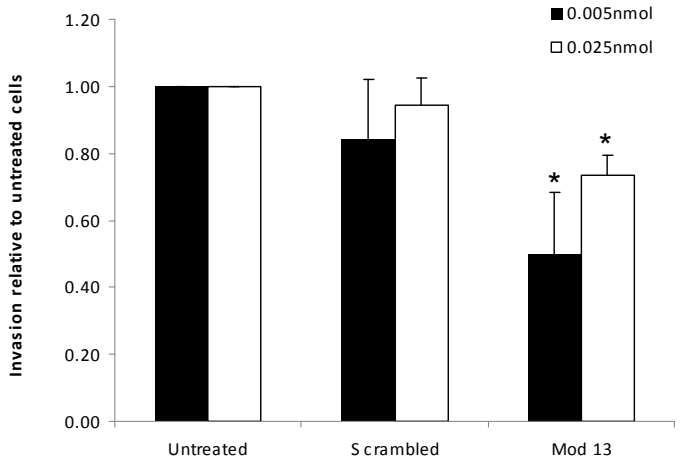
Figure 1. Production of RNA aptamers.
SS DNA template was used to create Mod 12 and Mod 13 aptamers (also called AIA 1 and AIA 2). RNA aptamers with T7 RNA polymerase. The RNA aptamers are indicated by the single strong intensity bands in the "+" lanes (arrow gives example). SS=single stranded DNA; "-" =No RNA polymerase; "+"= RNA polymerase' Ld = ladder.

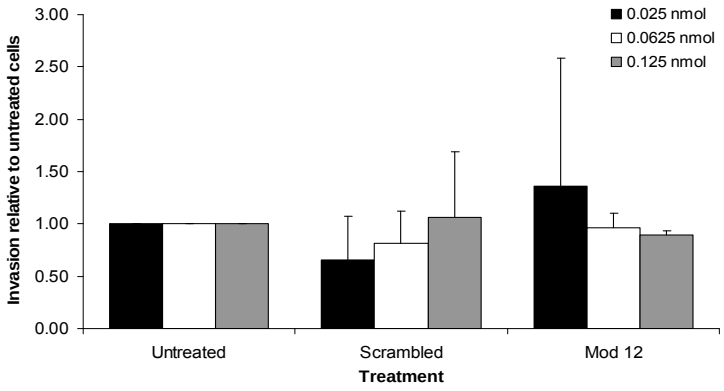


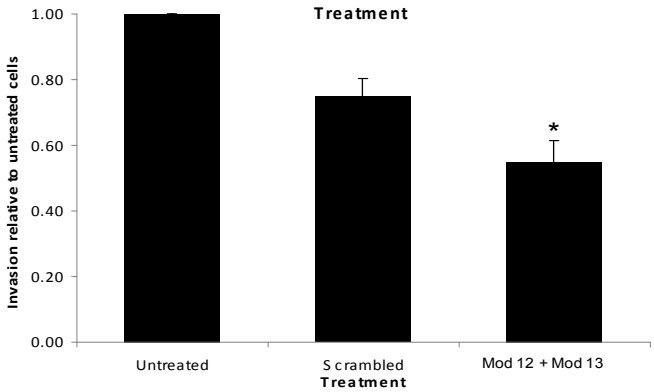
Task 1.1 Compare the anti-invasive aptamers (AIAs) efficacy and synergy on in vitro invasion.

We compared the effect of Mod 12 and Mod 13 and their combination on PC-3 luciferase cells. Mod 12 had minimal anti-invasive ability compared to scrambled control; whereas, Mod13 had marked anti-invasive ability (~50% reduction of invasive cells) compared to scrambled control (Fig. 2). The combination of both anti-invasive aptamers was less anti-invasive than Mod 13 alone.

Figure 2. MOD 13 has greater anti-invasive properties than MOD 12 in PC-3 cells.
PC-3-lux cells were plated in modified Boyden invasion chambers with scrambled aptamers and either Mod 12, Mod 13 or the combination of both at various concentrations. After 24 hours, the invasive cells were counted. Result are shown as relative to untreated cells. *p<0.05 versus untreated cells.



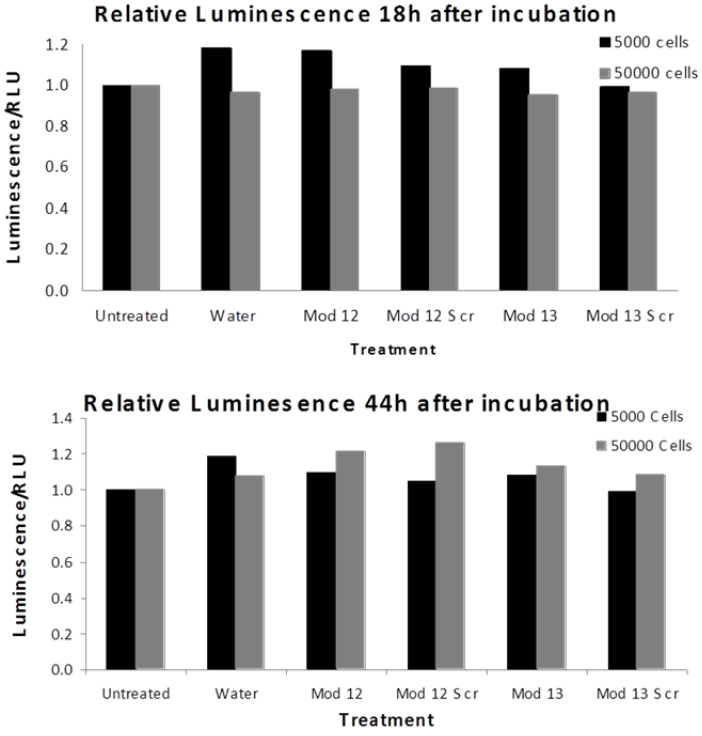




Task 1.2 Determine AIAs effect on Cell's growth.

In order to determine if the AIAs had a direct impact on cell growth, we incubated PC-3-luciferase cells with Mod 12 or Mod 13 and measured total cell number (as indicated by overall luminescence) at 18 hours and 44 hours after beginning incubation with AIAs. We also evaluated the impact with a low and high tumor cell density. Neither AIA had an impact on overall cell growth at either high or low cell density or at either time point (Fig. 3). These results indicate that the AIA's impact on invasion or metastasis is not through a direct impact on cancer cell growth. These results also indicate that there is no impact on cell proliferation or cell apoptosis. However, we cannot rule out that there are opposite effects on these parameters, which would result in no impact on overall growth.

Figure 3. Relative luminescence of cells following incubation with aptamer



Task 1.3 Determine if the AIA's anti-invasive effect is PCa specific or impacts other cancers.

We evaluated the effect of Mod13 on in vitro invasion of several other cancer types. It decreased the invasion of UC6, a bladder cancer cell line, but not of MDA-231 (breast cancer) (Fig. 4). It did decrease invasion of 82L osteosarcoma cells, which is a highly invasive variant, of the OS-187 osteosarcoma cells, which it did not decrease. This latter observation indicates that it successfully targets invasive cells; however, a caveat is that the scrambled aptamer also inhibited 82L and UC6 invasion. Thus, this may be a non-specific effect of aptamers. We then wanted to re-evaluate the in vitro invasion with our more precise method of quantification (see following paragraph on optimizing invasion assays). At this point, it appeared a specific anti-invasive effect is seen only in prostate cancer, as the aptamers can inhibit prostate cancer invasion above and beyond that of the scrambled controls.

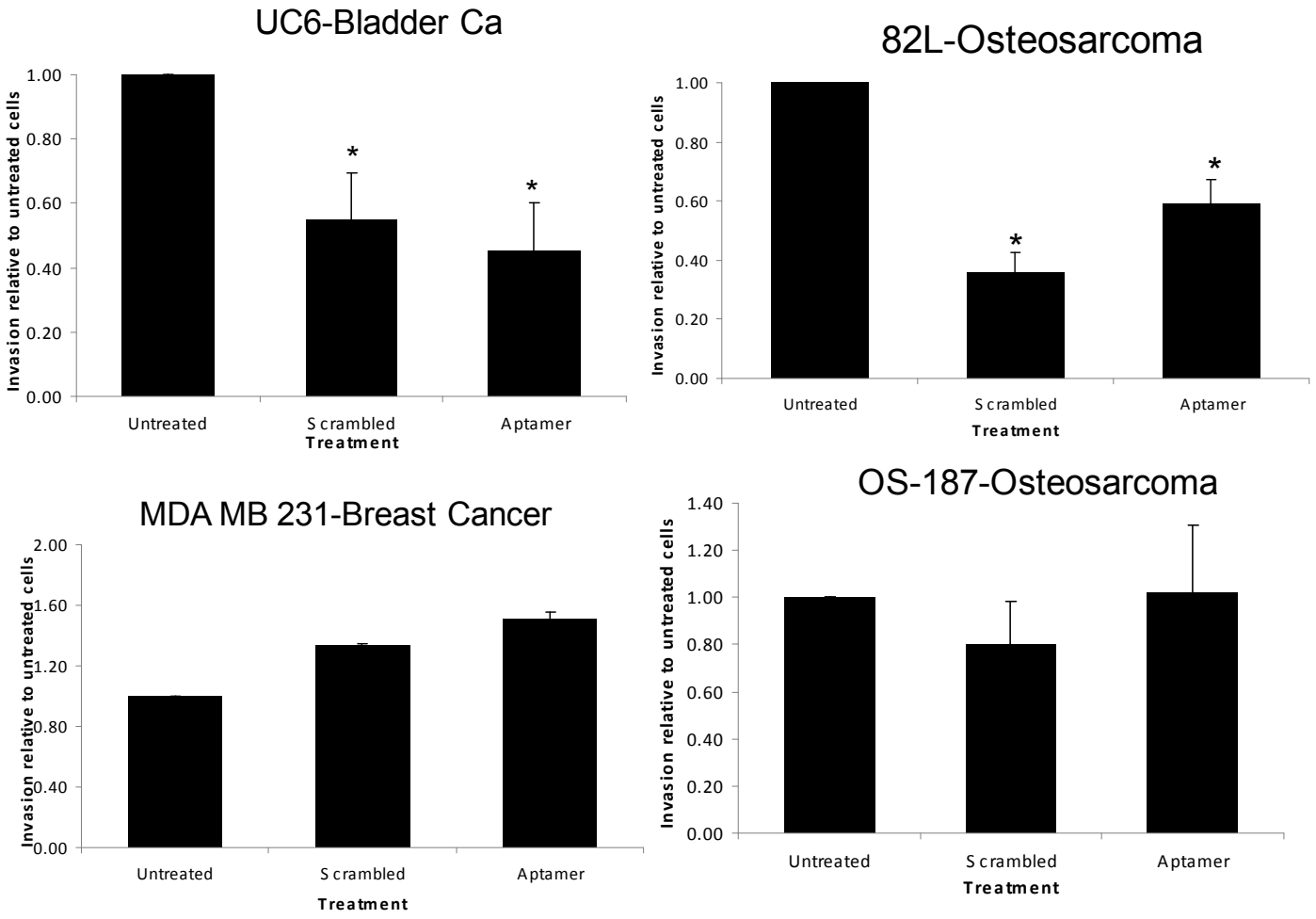


Figure 4. Effect of Mod 13 on in vitro invasion of various cancer cell lines.
Cells were plated in chambers of modified Boyden chambers and either untreated or treated with scrambled control or Mod12 aptamer (5 μg/ml). 24 hours later the numbers of invasive cells were quantifed. Data are presented relative to non-treated cells. *$p<0.05$ versus untreated cells.

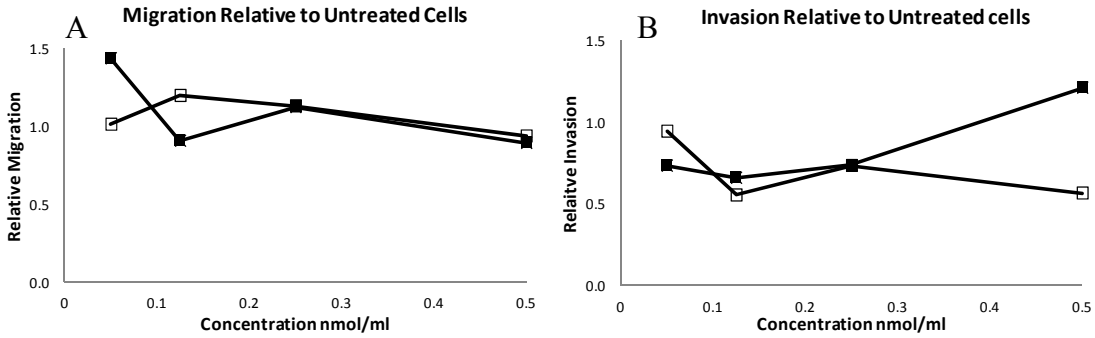
At this point, it appeared that we had an aptamer to perform further investigations; however, a new technician came into the lab to take over this project. The new technician, in preparation for continuing the study onto Aim 2, wanted to evaluate the aptamers in their hands. While they did see some modification of invasion, it was not as robust and consistent as we wanted as indicated below.

Reassement of analysis of RNA aptamersPrevious work had focused on RNA SELEX, including in vivo and in vitro experiments demonstrating an inhibitory effect on both invasion through a matrigel membrane and metastasis when injected into a mouse model. Work had been ongoing to identify the most suitable concentration to use and to determine other cell lines that were affected by the aptamer, but had previously been inconclusive

*Effect of different levels of aptamers on invasion and migration*We compared the effect of the Mod13 aptamer on PC-3luc cells to determine the optimum level of aptamers to use. No

significant inhibition of invasion or migration was observed compared to scrambled RNA (Fig. 5).

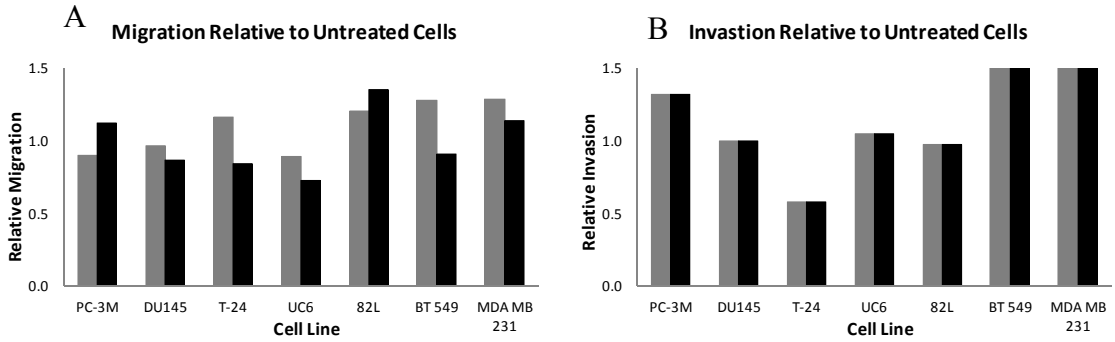
Figure 5. Invasion and migration relative to control cells.
0.5ml of cells at 2x105ml^{-1} were incubated overnight in a chamber with either a PET membrane or a matrigel layer over a PET membrane to determine invasion and migration. A) Relative Migration. B) Relative Invasion □Scrambled control; ■ AIA1.



Effect of Mod13 Aptamer on Other Cell Lines

Previous work had suggested that the ability to inhibit invasion was limited to prostate cell lines. This was extended to include further cell lines. No effect on these cell lines was observed (Fig. 6).

Figure 6. Invasion and Migration Relative to Control Cells of Different Cell Lines
0.5ml of cells at 2x105ml-1 were incubated overnight in a chamber with either a PET membrane or a matrigel layer over a PET membrane to determine invasion and migration. A) Relative Migration. No significant inhibition of migration through a PET membrane was observed compared to scrambled RNA. B) Relative Invasion. No significant inhibition of invasion was observed. ■ Scrambled control, ■AIA1 aptamer.

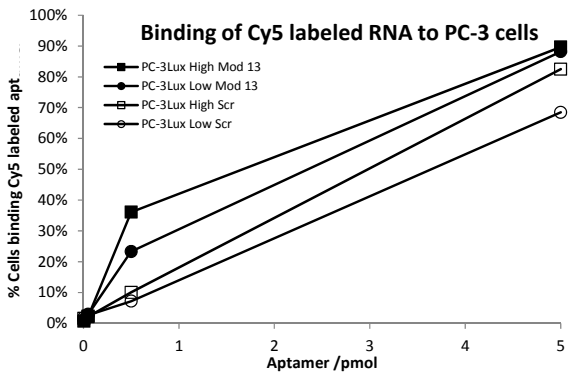


Determination of binding efficiency.

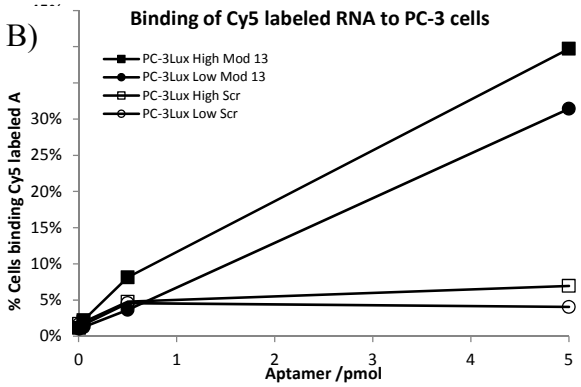
Previous work had shown binding of the aptamers to the cell lines, but no quantitative analysis had been performed. To determine binding , aptamers, scrambled control and the variable region of each were labeled with Cy5 and binding to different cell lines investigated.

Figure 7. Binding of labeled Aptamer

A)



B)



Cy5 labeled RNA was incubated with cells and gently washed. The percentage of cells with the bound RNA was measured with flow cytometry. A) Full aptamer sequence B) Variable region only

The results showed that although the aptamers sequence had higher binding than the scrambled RNA, there was no difference in its ability to bind the high and low invasive PC-3 luc. The variable region alone had higher binding, but showed no difference in binding of the Mod13 region and the scrambled region. It also did not discriminate between other cancer cell lines (data not shown).

It was unclear why the results were not repeatable but potential problems (not all inclusive) were (1) we had obtained a new production lot of aptamer and the original lot had some non-specific effect due to an additive; (2) the cells had changed their cell membrane targets as they grew in vitro (not an uncommon occurrence), (3) differences in technique among the different technicians, (4) inaccurate reporting or methods among the two technicians; however, review of the laboratory notes indicated accurate evaluation and assessment and reporting of results.

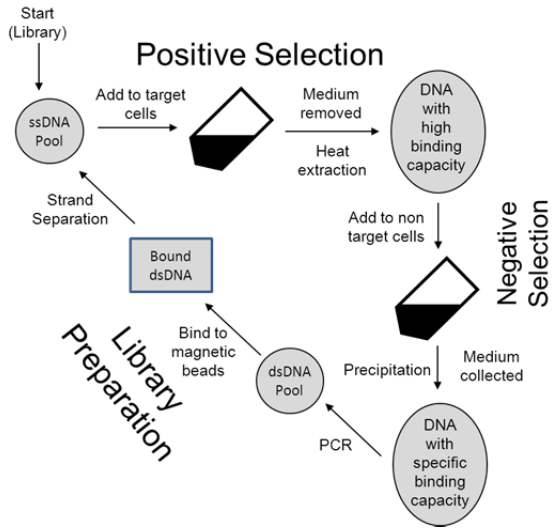
Due to the challenges of using the RNA aptamers and as the grant application reviewers had indicated that we should perform additional rounds of selection prior to proceeding we opted to develop a new set of aptamers for testing instead of progressing to Aim 2. Accordingly, we submitted a revise statement of work (SOW) in January of 2012, which was approved for the remaining grant period.

The SELEX procedure can used to create either DNA or RNA aptamers and both have been successfully used in the past, with their own advantages and disadvantages. Due to the relative stability of DNA aptamers, which would more likely translate into a clinical therapy, we elected

to pursue creating new anti-invasive aptamers using selection of DNA through the SELEX procedure. The literature also supports the use of DNA molecules to bind specific cells or proteins, including selecting on cancer cells with non tumorgenic cells as the negative selection cell.

Task1.4 Production of DNA Aptamer

Figure 8. SELEX Procedure.



An initial ssDNA library was mixed with target cells and incubated on ice for 45 minutes. The positive selection cells were washed to remove any DNA that was not bound to a target and then heated at 95C for 10 minutes to elute the DNA. This was then added to the negative selection cells and incubated again. The cells were washed to remove the non bound DNA, which was precipitated and used as the template for PCR, using a biotinylated primer. This was bound to magnetic beads and the non biotinylated strand recovered.

Throughout the SELEX procedure, approximately 100pmol ssDNA was used as the template. $1x10^6$ PC-3^{luc} high invasive cells, previously developed were used as positive selection unless otherwise stated and removed from plate without trypsin to leave cell surface protein molecules in as close to their native state as possible and to maximize the number of available protein sites for the aptamer to bind. Each stage apart from round 1, the aptamers bound to the positive selection eluted in 50µl and 25µl of this used for the negative selection.

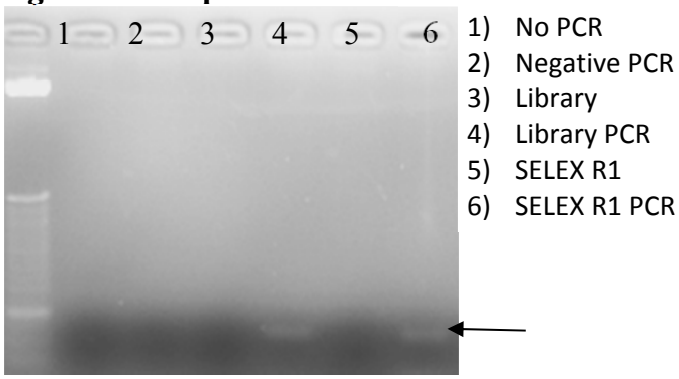
Previous studies have suggested that twenty rounds of SELEX may be necessary to isolate DNA sequences with high and specific binding affinity so a SELEX scheme was designed with the intent of performing at least fifteen rounds. This would increase the strength of the negative selection gradually, starting with a non cancerous prostate line, which was used to select against general prostate cell surface features that are not involved in the cancer phenotype. Secondly, rounds of SELEX would be performed with a prostate cancer line that has a poor metastatic potential in order that features associated with cancer but not metastasis would be selected against. By limiting the number of rounds using these cell lines, it was anticipated that the selection would not be complete, but that no DNA molecules that bound to cell surface features that were important in invasion or metastasis would be lost. Finally, SELEX as performed with a

line developed from the same parental source as the positive selection that differed only in its ability to invade through a matrigel membrane. This was expected to differ in only a few molecules and for these differences to be in terms of number of cell surface molecules rather than their presence or absence. By performing several rounds of SELEX before this cell was used, it was expected that any DNA bound to molecules would be present in high quantities that so it would not be lost by binding to the features on the cell with low invasive potential.

In addition to changing the negative selection cell, within each set of SELEX rounds, the stringency increased, starting from a low number of trypsinized cells, which would have lost some of the cell surface features, to cells that were removed without trypsin, so that the tendency to select aptamers that might be internalized was reduced. At this stage, we have performed 18 rounds of selection as detailed below:

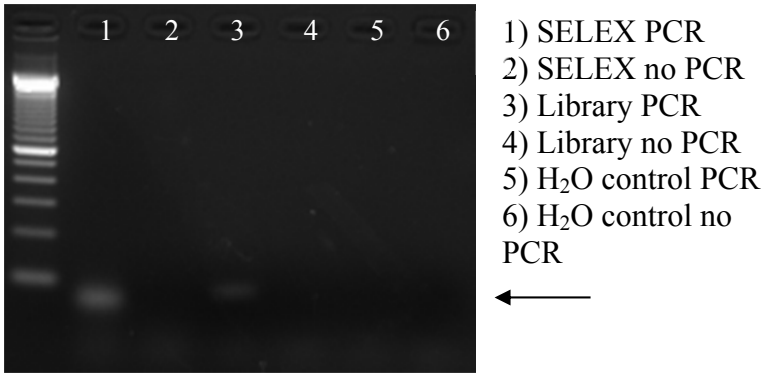
SELEX Round1, Positive selection only, $6x10^6$ cells (procedure: Fig. 8). Products were seen using either the initial library as template, or the SELEX round 1 precipitated DNA (Fig. 9). No band was observed without amplification, or with the negative control

Figure 9. PCR products of SELEX Round 1.



SELEX Rounds 2-18, Negative Selection. Negative selection was performed with the non cancerous prostate line BPH-1 in rounds 2-5 and with prostate cancer line LNCaP which has low invasive capabilities in rounds 6-10. In rounds 11-18, negative selection was performed with the PC-3^{Luc} low invasive line developed previously. Bands were observed with the positive control, using the initial library and with the R17 precipitated DNA (Fig. 10). No bands were seen with negative control or non-amplified DNA, while SELEX R17 product was a discrete single band.

Figure 10. SELEX R17 PCR products.



Analysis of sequences developed through SELEX procedure

In order to determine the success of the SELEX procedure and if further rounds were likely to be required, sequencing was performed on clones from round 12 and round 15,both of which had used PC-3^{luc} low invasive cells as the negative selection. The results showed that the SELEX procedure was yielding sequences that were more highly represented than would be expected by chance alone (Fig. 11).

Figure 11. Sequences from SELEX procedure.

A	**Sequence**	**Length**
1	TGTGGTGCGTTGGTGCATTTGTTTGCCTGTGGTTTGCTTGTTCGC	45
2	TGTGCGTGCTGTTTGCGTCTGTTGCTTTTTGTTGGTAATGTGGTG	45
3	TCTTTGTTCCTTGTCTGCTTTTTTGTGAGTGGTCTTGGTCGTGTG	45
4	TGCGTTGTGTTCTTCTTGTTTCATGTTATTCGTGTCCTCCGTTGT	45
5	TGTTGCGTAGCGTTGCTTCCTTGCATGTTGTGGTGTGTGTGTTG	44
6	CCACTGCGGATCCCATTCCCGTGCTTTTGC	30
7	CTGCGAGTGGTGTGTGTTCCGTGC	24
7	CTGCGAGTGGTGTGTGTTCCGTGC	24
8	TGAGGTTGCCGTTGCTTTGTG	21
9	CCTCGTTGGCGTGCGGTGCTT	21
9	CCTCGTTGGCGTGCGGTGCTT	21
10	TGTTGGTGTTGTTTTGGCT	19
11	GCCCACATCGCTCCTACA	18
11	GCCCACATCGCTCCTACA	18
B	**Sequence**	**Length**
1	TTGGTGCTTGCCTGTCCCTTCTACGTTTGTGCGAATGCC	39
2	TGCTATTGGTGGTCTTGTGTGGTGTTTTGGTGTG	34
3	TGCGTTGTACGGTACTTCTTTCCACGTGCGTTCC	34

4	TGTTGCTGGGATTAGCGCGTCTTCGTGGTTGTG	33
5	TGTTGCGCTGGTCAGTTTTGTCGTGTGTGTA	31
6	TGCACGATGCTTGGTTGTTTGTTGATCGG	29
7	TGCGCTGGATGTGTATGTTTGGCTG	25
8	CTGCAGTGCGTACATT***C***CGTTGCTC	25
8	CTGCAGTGCGTACATT***T***CGTTGCTC	25
9	CTGCGAGTGGTGTGTGTTCCGTGC	24
9	CTGCGAGTGGTGTGTGTTCCGTGC	24
9	CTGCGAGTGGTGTGTGTTCCGTGC	24
9	CTGCGAGTGGTGTGTGTTCCGTGC	24

Sequencing Results. A) Round 12, B) Round 15.
Primer sequences omitted for simplicity. After twelve rounds, three of the sequences returned were present in duplicate. After fifteen rounds, only one sequence was present more than once, in four out of the thirteen clones returned, but this was also present in the round twelve sequence results (Fig. 12). There were two other sequences that differed in just one nucleotide from each other and also showed considerable nucleotide sequence alignment with the highly represented sequences

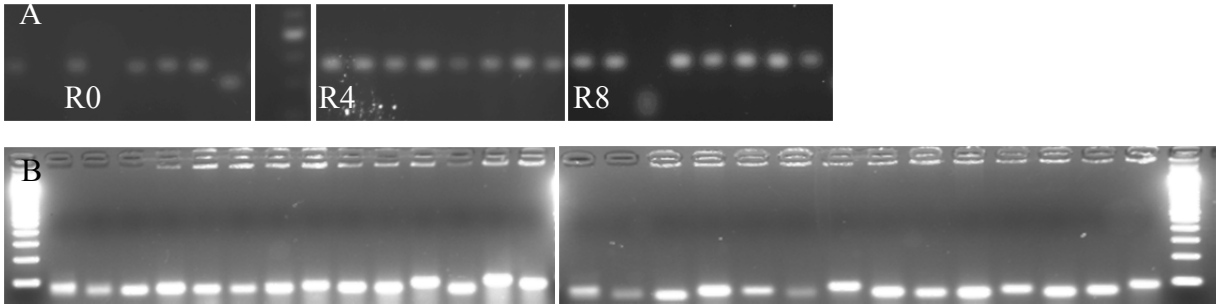
Figure 12. Sequence Alignment of Round 15 Sequences

```
CTGC~AGTGCGTACA~TTCCGTTGCTC~~
CTGC~AGTGCGTACA~TTTCGTTGCTC~~
CTGCGAGTGGTGTGTGTTCCG~TGC~~~
CTGCGAGTGGTGTGTGTTCCG~TGC~~
CTGCGAGTGGTGTGTGTTCCG~TGC~~
CTGCGAGTGGTGTGTGTTCCG~TGC~~
```

At this stage it was decided to use the highly represented sequence for further analysis, but also to continue with further rounds of SELEX.

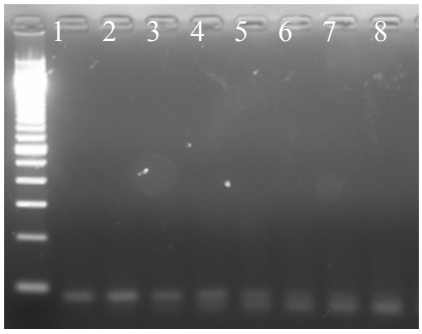
An unexpected feature of the sequence results was that the though the library had a 45 base pair random sequence, after twelve rounds, only four of the fourteen returned sequences were full length and two were as short as eighteen. After fifteen rounds, all the returned sequences were between twenty four and thirty nine bases long. To determine the extent of the variability in sequence length, SELEX DNA from the library, round 4 and round 8 was cloned and colony PCR performed. R12 and R15 had already been shown to have fragments of different sizes, but a selection of R15 clones were amplified to give a better idea of the range of sizes

Figure 13. PCR of clones from different rounds of SELEX.
SELEX DNA from the library, round4, round8 and round 15 was amplified by PCR using non biotinylated primers and cloned into PCR2.1 TOPO. Colony PCR was then performed using the same primers and the result run on a gel. A) R0, 4 and 8. One clone from the amplified library showed a smaller size. All the round 4 clones and R8 clones that successfully amplified were the same size as the library PCR, 85bp. B) R15, twenty eight clones were selected and amplified showing a wide range of product sizes



PCR of the pool of DNA for each round showed what was apparently a single band and a discrete product. To determine if there was a PCR bias, a mixture of library and R18 amplified together. No evidence of preferential amplification of shorter sequence was detected.

Figure 14. PCR of mixtures of library and Round 18 product.
ssDNA library and R18 product at equal concentrations were mixed at different ratios and amplified. Lane 4, with 6µl library (full size) and 4µl R18 and lane 5 (4µl Library and 6µl R18) show similar amplification.



1) 1µl library
2) 9µl library + 1µl R18
3) 8µl library + 2µl R18
4) 6µl library + 4µl R18
5) 4µl library + 6µl R18
6) 2µl library + 8µl R18
7) 1µl library + 9µl R18
8) 10µl R18

After 18 rounds of SELEX, the library was cloned and sent for high throughput screening.

Figure 15. Most common sequences from the round 18 library

Copies	Variable Region	Length
649	CTGCGAGTGGTGTGTGTTCCGTGC	24
417	CCACTGCGGATCCCATTCCCGTGCTTTTGC	30
164	TGCACGATGCTTGGTTGTTTGTTGATCGG	29
109	TGCTTGGACCGGTTTGGTGACTCCCTGTG	29
87	TGCACCCTACTCCTGCTTTTGCTGTGCCC	29
87	TGCGCTGGATGTGTATGTTTGGCTG	25
80	TGCTGCATGCGGTTCTGCTCTGGT	24
78	CTGCAGTGCGTACATTCCGTTGCTC	25
68	ACACACGTGTGGTTCTTGCTGTG	23
64	GTCAAGCGGGTGTGTGTTTGGTG	23
62	GACGGTGCATTGGTGTGTGTTTGC	24
58	CCACTGCGGATCCCATTCCGTGCTTTTGC	29
56	CCACTGCGGATCCATTCCCGTGCTTTTGC	29

Primer sequences omitted for simplicity

Most commonly observed sequences out of over 9000 identified. Approximately 80 sequences were present at least 10 times. The most common sequence had previously been identified in both round 12 and round 15, and second most common sequence in round 12.

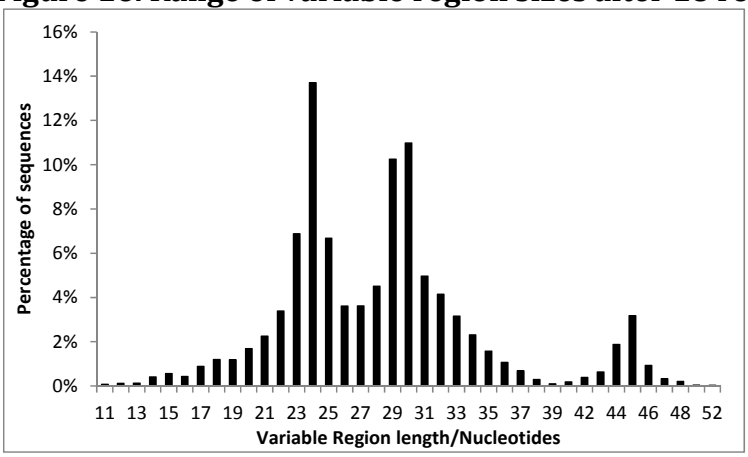
Many sequences were closely related and there were two variants of the second most common that each had more than 50 copies. The most common sequences were present at a relatively low frequency, less than 10% of all sequences. There was a bias in the representation of bases (Table 1).

TABLE 1. Base frequency

Base	**Frequency**
T	38.7%
G	32.1%
C	22.6%
A	6.7%

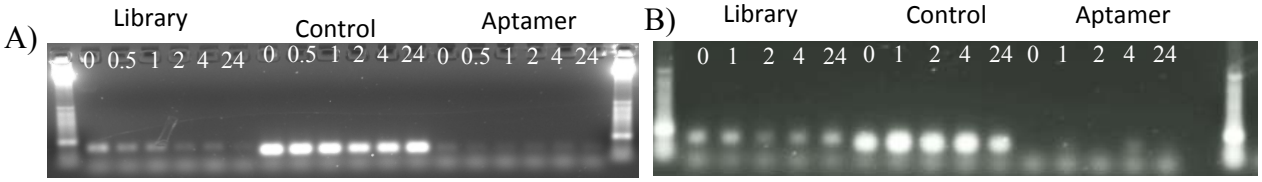
Determining range of variable region. Due to the variation in length of the apatamers, we wanted to determine if there were hot areas of variance and how extensive this was. This could indicate non-critical areas and highlight the non-variable areas. Approximately 9000 sequences were analyzed and the length of the variable region determined. The peak at 24 represents the most common sequence and variants (Fig. 16). The peak at 45 represents the original length DNA and shows a roughly normal distribution

Figure 16. Range of variable region sizes after 18 rounds of SELEX



Stability of aptamer. In order to determine if the aptamer was impacted by biological fluids we incubated in conditioned media for various time points up to 24 hours and compared its levels of PCR amplification to that of aptamer in plain media. There was no difference with Media. Conditioned Media had less DNA, but the results were not conclusive.

Figure 17. Stability of Aptamer DNA



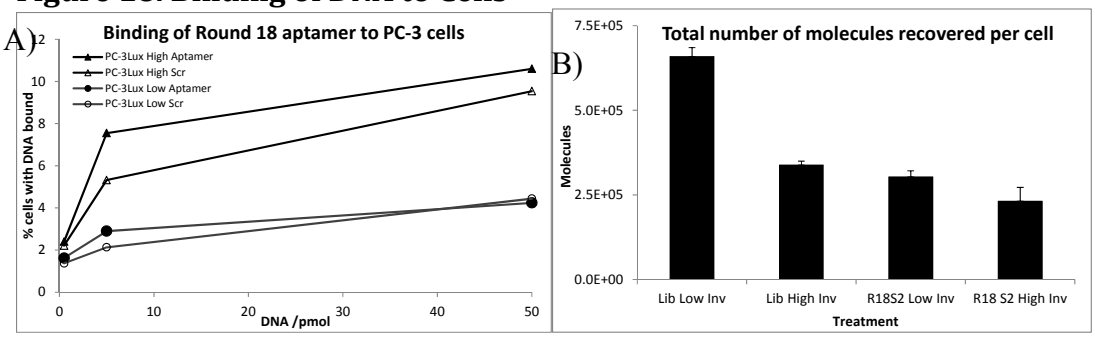
10pmol R18 seq2, library or control scrambled DNA was incubated for indicated time points (hrs) in 100µl in a well of a 96 well plate in triplicate in A) Media or B) Conditioned Media. It was precipitated overnight and resuspended in 100µl water. 1µl was amplified for 15 cycles

Effect of Aptamer on cells

The ability of the DNA to bind to the cells was then investigated. Two methods were used, firstly direct measurement to see how well the DNA binds. Secondly, an indirect method to determine how much DNA can be recovered from washed cells.

The highly invasive cells showed more binding to the highly invasive than the low invasive cells, when measured by flow cytometry, but this was not specific for the round18 product compared to control DNA (Fig 18A). The amount of DNA recovered from cells was not statistically different for the low and high invasive cells with the Round 18 sequence. (Fig. 18B) The reason for this disparity was not clear, but it may be related to the elution method or to the different treatment the cells received in each experiment.

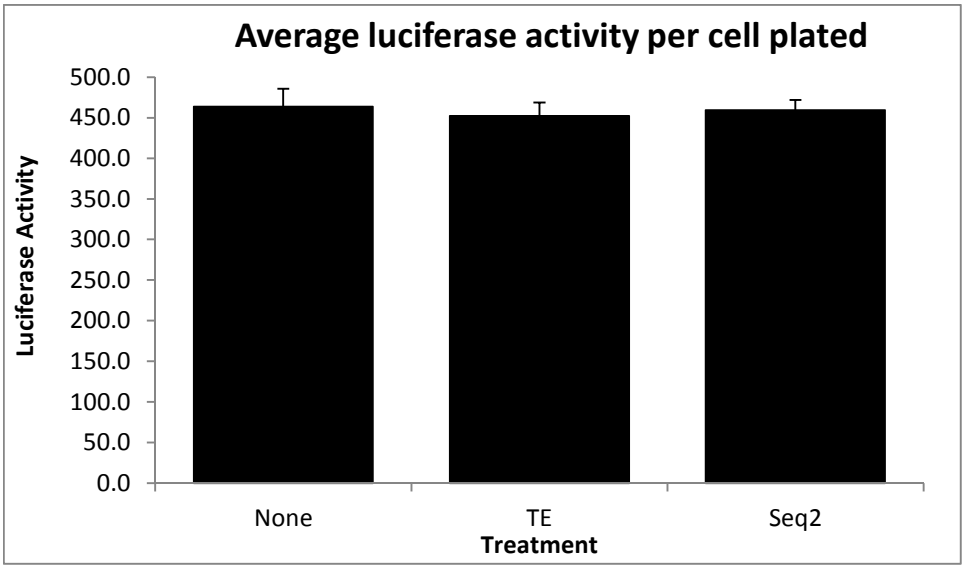
Figure 18. Binding of DNA to Cells



A). Fluorescein labeled DNA from SELEX round 18 was incubated with PC-3 cells and unbound DNA washed away. The proportion of cells with DNA bound to them was determined by flowcytometry. B) Library or R18S2 (sequence 2) mixed with cells and cells washed. After washing, bound DNA eluted and number of molecules recovered estimated by qPCR.

To rule out if any effects on invasion or migration we might observe could be in part due to an effect on viability, the aptamer was tested for any toxicity effects by incubating cells with apatamers. No toxicity was observed (Fig. 19).

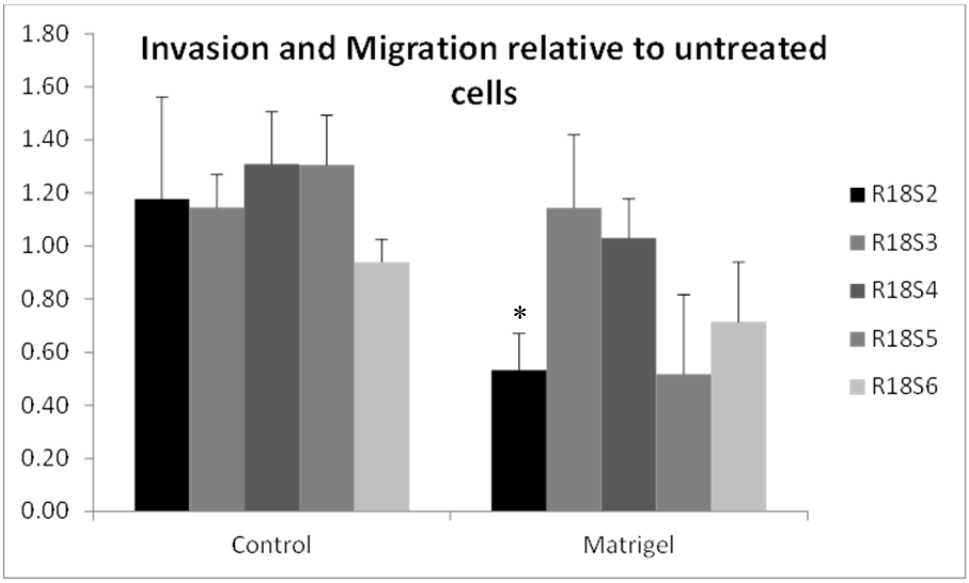
Figure 19. Effect of aptamer on cell viability. $1x10^4$, $5x10^4$ and $5x10^5$ PC3 prostate cancer cells were plated in wells of a 96 well plate in 100µl media and 0.5nmol aptamer added per well. Luciferase activity was measured the following day and the activity per cell plated calculated



Effect on in vitro invasion. We next assessed if aptamer had an impact on in vitro invasion. This was performed using modified Boyden chamber assays. Aptamers had no effect on general

migration (Fig. 20 Control). The R18S2 aptamer inhibited PC-3's invasive ability through Matrigel; whereas, other apatamers did not have a statistically significant effect (Fig. 20)

Figure 20. Effect of Aptamer on Invasion and Migration PC-3 cells were plated in chambers of modified Boyden chambers and either untreated or treated with scrambled control or aptamer (1nmol/ml). 24 hours later the numbers of invasive cells were quantified. Data are presented relative to non-treated cells. *P=0.017 versus control R18S2.



Summary of DNA SELEX

A procedure was developed to obtain DNA aptamers through a SELEX method. Sequencing of a small number of clones was performed at rounds 12 and 15 which showed that some sequences were increasing in frequency, but that the length of the variable region was decreasing. This was confirmed by amplification of product from each round, which showed the length to be constant for the first 10 rounds then to drop consistently. High throughput sequencing was also performed after 18 rounds, which yielded almost 3000 variable regions out of almost 10000 sequences. Most sequences were present at a low frequency and only 13 sequences were detected over 50 times. The most common accounted for around 8% of the sequences. Two observations raised questions about possible biases in the selection procedure. Firstly, after 10 rounds, the length of the product began to decrease, such that at 18 rounds, only 3% of the sequences were still the original 45 nucleotides long, and only 7% were between 42 and 48 nucleotides. The second concern was that after 18 rounds, adenine accounted for only 6.7% of all the bases observed. We were not able to discover the reason for this and preferential amplification of shorter sequences was not observed.

Five of the most common sequences from round 18 were selected for further testing. Three of them did not show any ability to inhibit cell invasion of migration across a matrigel membrane. Two sequences had some suggestion of this ability, but it was not consistently observed. The sequence with the best results was further tested and not seen to be able to discriminate between the PC-3^{Lux} high invasive and PC-3^{Lux} low invasive cells in a binding assay, but was able to when measured by flow cytometry, but in this instance, binding was no better than a randomly

generated sequence and was less than 10% of the cells. Recovery of the DNA after overnight suggested that it was stable in in conditioned media. One of the aptamers, R18S2, inhibited in vitro invasive ability of PC-3 cells indicating we have identified an anti-invasive aptamer.

KEY RESEARCH ACCOMPLISHMENTS

- Determined that although the initial RNA aptamers were effective, the effect was not robust.
- Developed DNA SELEX method
- Accomplished 18 rounds of SELEX
- Identified several DNA aptamers that had increased representation from the SELEX procedure
- Identified a DNA aptamer that inhibitsin vitro invasion of PC-3 cells

REPORTABLE OUTCOMES:

Poster: Novel Application of Aptamer Selection (Pheno-SELEX) to Target the Invasive Phenotype Successfully Creates Anti-Metastatic Aptamers. DOD Impact meeting. 3/2011

Video: DOD Impact meeting highlights:
http://cdmrp.army.mil/pubs/video/pc/impactvideo_keller.shtml

Seminar: Targeting cancer invasion using apatamers. Nanjing Medical University, Nanjing, China, June 2012

Product: Creation of anti-invasive aptamers

CONCLUSION:

Our original goal was to explore the role of RNA aptamers in targeting prostate cancer invasion. However, during the performance of this project, it became clear our original RNA aptamers were not consistently effective at binding to and inhibiting invasion of PCa cells. For this reason and also based on comments of the reviewers of the original proposal, we opted to reisolate aptamers; however we changed to using DNA for the aptamer material as it DNA had the potential to have greater stability than RNA. So a new Statement of Work was submitted and approved to perform this activity. To that end we performed SELEX using a DNA aptamer library and identified several aptamers that were highly represented after 18 rounds of SELEX cycles. Several of these aptamers appeared to inhibit in vitro invasion of PC3 prostate cancer cells. These results provide (1) proof of concept that without a priori knowledge of a target, aptamers can be created that inhibits a function; and (2) potential therapeutic molecules for inhibition of PCa invasion. Although, the results appear promising, certain limitations must be

considered including ensuring an appropriate negative selection control is used to optimize removal of any non-active aptamers. Also, the material (RNA vs. DNA) needs to be given careful consideration. This work is important as it establishes the groundwork for additional cell-based SELEX methods to target other cancer-related properties.

REFERENCES:

None

APPENDICES:

DOD IMPACT ABSTRACT

Targeting the invasive phenotype using systemic evolution of ligands by exponential enrichment creates anti-metastatic aptamers.

Evan T. Keller, Greg Shelley, June Escara-Wilke, Jill Keller

Background and Objectives: Most men with advanced prostate cancer (PCa) die due to metastatic disease. Although therapeutic gains against primary tumors have been achieved, there is a dearth of effective therapies to prevent or diminish metastasis. Multiple methods (e.g., small molecules and antibodies) have been designed to target specific proteins and signaling pathways in cancer. However, many mediators of the metastatic phenotype are unknown and the ability to target these phenotypes would help mitigate metastasis. Aptamers are small DNA or RNA molecules that are designed for therapeutic use. Design of aptamers to target cancers can be challenging. Our goal was to determine if we could design aptamers that target a phenotype of metastasis, i.e., invasion.

Methods: A modification of systemic evolution of ligands by exponential enrichment (SELEX) was used to target the invasive phenotype of PCa cells. We call this method pheno-SELEX. A highly invasive PCa cell line was developed using in vitro selection in invasion assays. The cell line was then used to identify aptamers that bound to it with high affinity as opposed to a less invasive variant to of the cell line. The anti-invasive aptamer (AIA1) was then tested for its ability to inhibit in vitro invasion and in vivo metastastasis in a murine model of PCa.

Results: AIA1)was found to inhibit in vitro invasion of the original PCa cell line, as well as an additional PCa cell line and an osteosarcoma cell line. AIA also inhibited in vivo development of metastasis in both an intracardiac injection metastasis model of PCa and an orthotopic (i.e. injection into the tibia) osteosarcoma model of metastasis.

Conclusions: These results indicate that pheno-SELEX can be successfully used to identify aptamers without knowledge of underlying molecular targets. This study establishes a new paradigm for identification of functional aptamers.

Impact statement: Aptamers designed using pheno-SELEX could accelerate therapies, including anti-metastatic and other cancer-related phenotypes, without the need to know the underlying targets. This could result in creating therapies prior to dissecting the complex molecular pathways of cancer. Furthermore, the aptamers could be used to identify the targets to which they bind for development of additional specific therapies.